ON THE LEUCOCYTOTOXINS OF NORMAL SERUM.*

HERBERT M. GOODMAN

(From the Memorial Institute for Infectious Diseases, Chicago.)

IT has long been known that normal sera may contain elements toxic to cells of other animals, as exemplified by the normal hemolysin or the less familiar haptin producing necroses (Uhlenhuth,[1] Pfeiffer[2]). The occurrence in sera of bodies possessing a toxic action on foreign leucocytes has been observed, but very little is known of their nature. Noguchi[3] found that the leucocytes of certain species of crabs are injured by the serum of a number of poikilothermous animals. Christian,[4] studying a considerable number of combinations, detected leucotoxic action in only one case. He placed the leucocytes in a warm chamber under the microscope and added the serum to be tested. Loss of ameboid movements was taken to indicate the toxic action. Then there are the rather conflicting observations of Laschtschenko[5] and of Metchnikoff,[6] those of the former to the effect that the injurious effect of foreign sera upon leucocytes is prevented by heating to 60°, and those of the latter indicating that sera heated to 60° still retain the power of agglutinating leucocytes. Also Ruediger and Davis[7] state that human leucocytes are injured by the sera of many species of poikilotherms and that this action is prevented by previously heating the serum to 55° C.

In place of the method of Christian, the leucocytes in my experiments were examined for signs of degeneration by means of stained films. Leucocytes were obtained either from the exudate caused by intrapleural injections of aleuronat or from blood cream, washed in NaCl solution, and usually 0.2 c.c. of fairly dense suspensions (unless otherwise stated) were mixed with varying quantities of the serum tested, the whole being made up to 0.5 c.c. with normal NaCl solution and incubated at 37° C. for about one hour. Smears were then made and stained with thionin.

* Received for publication February 29, 1908.

[1] *Ztschr. f. Hyg.*, 1897, 26, p. 384.

[2] *Ibid.*, 1905, 51, p. 183.

[3] *U. of Penn. Med. Bull.*, 1902, 15, p. 295.

[4] *Deut. Archiv. f. klin. Med.*, 1904, 80, p. 333.

[5] *Arch. f. Hyg.*, 1900, 37, p. 290.

[6] *L'Immunité*, Paris, 1901, p. 199.

[7] *Jour. Infect. Dis.*, 1907, 4, p. 333.

In contrast to the leucocidin of *Staph. pyogenes aureus* (Van de Velde[1]), whose action is limited to the cytoplasm of the white corpuscles, the action of normal serum leucotoxin is principally upon the nucleus, so that it appears to be more closely allied to the leucocidin produced by *B. pyocyaneus* (Ghéorghiewsky[2]) and those formed by *B. anthracis symptomatici* and *B. edematis maligni* (Eisenberg[3]). Corresponding to the amount of serum employed and to the length of the incubation period the action of this body manifests itself in varying grades o

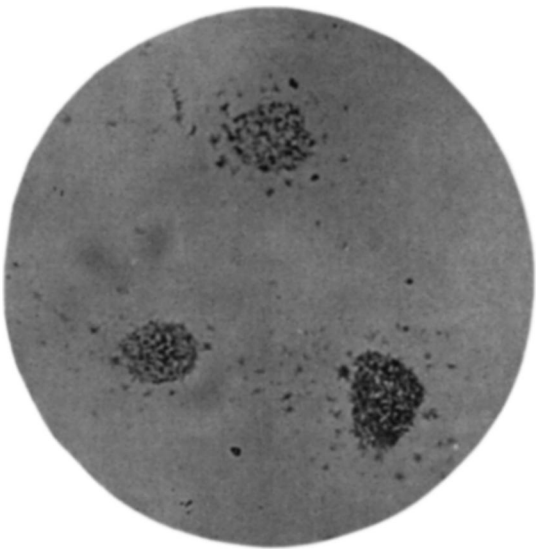

FIG. 1.—Nuclei disintegrated.

intensity ranging from almost complete destruction as shown by transformation of the leucocyte into a clump of granular débris, which stains a faint reddish brown with thionin, to a condition in which the nuclei merely lose their normal contour and become vacuolated and diffused over the entire cell; this change is accompanied by an alteration in the staining reaction, the nuclear material having a reddish tinge in place of the normal deep blue (see Figs. 1, 2, and 3). The change in the staining reaction may be obscured, however, by prolonged application of the thionin, which now may give the normal blue

[1] *Ann. de l'Inst. Past.*, 1896, 10, p. 580.

[2] *Ibid.*, 1899, 13, p. 298.

[3] *Compt. rend. Soc. Biol.*, 1907, 62, p. 491.

color. That both these types of degeneration are but different manifestations of the same phenomenon and are due merely to variations in the completeness of the reaction is shown by the presence of every conceivable intermediate grade between the two extremes in different smears. In all cases, however, the leucocytes in a single smear are in the same condition.

When the action is very marked all types of leucocytes are equally affected, but when it is less strong the cells show a variable suscepti-

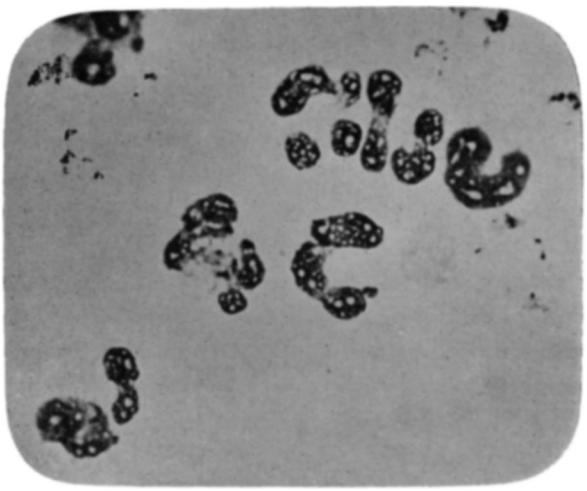

FIG. 2.—Vacuolization of nuclei.

bility to the poison, that of the large mononuclear leucocytes being greatest, that of the polymorphonuclears least, and that of the lymphocytes intermediate. A smear may thus show complete destruction of the large mononuclear leucocytes, distinct degeneration of the lymphocytes, and perfectly normal polymorphonuclear cells. This difference in the susceptibility of the various types of leucocytes is of some interest in view of the present conception of the adaptation of the large mononuclear leucocytes as devoted to immunity against animal cells especially and of the polymorphonuclears against vegetable cells (bacteria). In the experiments recorded subsequently + indicates mere degeneration of the large mononuclear elements with no visible change in the other types, + + destruction of the large mononuclears

with degeneration of the lymphocytes, and +++ destruction of all the leucocytes.

The first step was to determine how widely normal serum leucotoxin is distributed. Christian, using loss of ameboid movements as the criterion, found but a single instance in which leucotoxin was present, namely in chicken serum for dog leucocytes. It is true that Noguchi observed leucotoxins in the blood of several poikilothermata for the white cells of crabs, and Ruediger and Davis for human

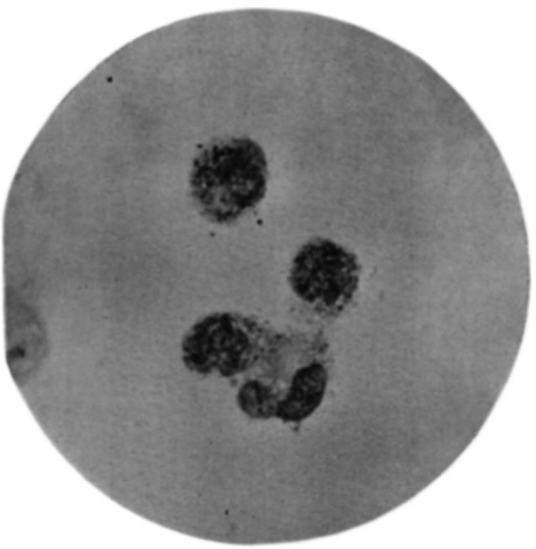

FIG. 3.—Nuclei degenerated and diffuse—sharp outlines lost.

leucocytes, but no further details are given by these authors. In order to determine whether the results of Christian's experiments would dovetail with those obtained by means of the staining reaction, four of the combinations employed by that investigator—chicken serum with human, dog, and rabbit leucocytes, and dog serum with rabbit leucocytes—were repeated. In each case the results tallied with those Christian obtained by the motility test. A number of additional combinations were then studied, and the results of which are recorded in Table 1. This shows that while normal leucotoxins are not widely distributed, they nevertheless are far from being as rare or unusual as might perhaps be surmised from the earlier observations, and consequently a knowledge of the nature of these

bodies is of importance. Unless otherwise designated the following experiments were performed with human serum and dog leucocytes.

TABLE 1.

DISTRIBUTION OF NORMAL LEUCOCYTOTOXINS.

SERA	LEUCOCYTES				
	Human	Dog	Rabbit	Guinea-pig	Swine
Human		+	−	−	+
Dog	−		−	+	
Rabbit	−	− (?)			
Guinea-pig	−	−			
Swine	−	−	−		
Goat	−	−	+	−	
Limulus	−	+ (?)			
Chicken	−	+	−	+	
Sheep	+	−	+	+ (?)	
Horse	−	+			
Beef	−	−			
Rat	−	+			

Leucotoxin is bound by the leucocytes, since on mixing an equal quantity of serum with a thick suspension of washed leucocytes and incubating at 37° C. for one hour, the supernatant fluid obtained by centrifugation is devoid of any toxic action on a fresh suspension of leucocytes. It is therefore possible to remove the leucocidal body from serum by saturation with leucocytes.

As noted, Laschtschenko and Metchnikoff have published conflicting statements concerning the thermolability of leucotoxin. I have found that it is invariably destroyed by exposure to 55° C. for 30 minutes.

By dilution of leucotoxic serum a quantity of serum may be obtained so small that it is incapable of producing toxic effects on a leucocytic suspension. If, however, this quantity of normal serum, too small to kill leucocytes by itself, be added to a larger quantity of serum, previously heated to 55° C. for 30 minutes, and which also is innocuous by itself, the mixture readily destroys white corpuscles exposed to its action (Table 2). Hence, the leucocidal action of normal serum is produced by the interaction of two independent bodies, one thermolabile and capable of acting in very minute quantities, the other thermostabile and less abundant or less active than the thermolabile element.

A question of the interest in regard to the nature of normal leucotoxin is that of its identity with normal hemolysin. The susceptibility

of both to separation into a complemental and an amboceptor-like fraction would seem to speak in favor of their identity. It appears

TABLE 2.

REACTIVATION OF HEATED LEUCOCYTOTOXIC SERUM.

Leucocytic Suspension	Normal Serum	Heated Serum	Result
0.2 c.c.	0.2 c.c.		+++
0.2	0.1		+++
0.2	0.05		+++
0.2	0.025		++
0.2	0.0125		++
0.2	0.006		+
0.2	0.003		−
0.2	0.0015		−
0.2	0.0007		−
0.2	0.00035		−
0.2		0.2 c.c.	−
0.2		0.1	−
0.2	0.003	0.1	+++
0.2	0.0015	0.1	+++
0.2	0.0007	0.1	++
0.2	0.00035	0.1	++
0.2			−

justifiable, however, to assume that the two bodies are quite distinct and independent of each other in view of the following facts. In the first place, the distribution of leucotoxins as shown above is not co-extensive with that of the normal serum hemolysins. This fact by itself might be considered ample proof that the two are distinct bodies. It is not entirely conclusive, however, for it is conceivable that the leucotoxic and hemolytic actions are two functions of the same substance, but that in some cases the former does not manifest itself because of some species peculiarity in the binding group of the leucocytes. In the second place, there is a quantitative difference in the minimum leucotoxic and hemolytic doses of serum for the blood elements of the same animal for it was found that the former manifests itself in far higher dilutions than the latter. This is shown in Table 3. The non-identity of the two substances is apparently indicated by this

TABLE 3.

QUANTITATIVE RELATIONSHIP BETWEEN NORMAL LEUCOCYTOTOXIN AND HEMOLYSIN.

Serum	Hemolysis (1 c.c. 5 per cent Susp.)	Leucolysis (0.2 c.c. Susp.)
0.2 c.c.	+	+++
0.1	+	+++
0.05	+	+++
0.025	+	+++
0.0125	−	++
0.006	−	++
0.003	−	+
0.0015	−	−

observation, but here again it must be admitted that the experiment is not conclusive and the result must be regarded as merely presumptive evidence. While the erythrocytes were used in 5 per cent suspensions of which 1 c.c. was taken and may reasonably be assumed to represent a fairly constant quantity of cells, this is not true of the leucocytic suspensions, for there is no way of obtaining a suspension of leucocytes equal in cellular richness to that of the erythrocytes, and there is also daily variation in the leucocytic concentration of the aleuronat exudate from the same animal. But even if leucocytic and erythrocytic suspensions of equal concentration were employed it is not impossible that a given quantity of one and the same amboceptor might be capable of sensitizing many times as many white as red cells.

Far more conclusive results are obtained by absorption experiments. By saturating a quantity of heated serum with an excess of red blood corpuscles the presence of an unbound specific leucotoxic amboceptor may be demonstrated by exposing white corpuscles to the residual fluid and adding a quantity of normal serum too small in itself to produce leucocytic karyolysis, and hence containing complement but no amboceptor, as proven by the control experiment in which the same quantity of the same normal serum manifests no action on an equal quantity of the same leucocyte suspension. The results of such experiments were strikingly positive. An example follows:

A 5 per cent suspension of red corpuscles 1 c.c. + heated serum 0.05 c.c.; place at 37° C. for one hour; centrifugate.

Supernatant fluid + leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = leucolysis.

Leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = no leucolysis.

Reversing this experiment, i. e., saturating the heated serum with leucocytes and using hemolysis as the indicator of reaction, gives results of identical significance.

Leucocytic suspension 1 c.c. + heated serum 0.03 c.c.; place at 37° C. for one hour; centrifugate.

Supernatant fluid + 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.015 c.c. = hemolysis.

A 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.015 c.c. = no hemolysis.

The conclusion, therefore, is that the thermostable element of normal leucotoxin is distinct from that of normal hemolysin.

An experiment was made to determine if the thermolabile body necessary for leucotoxic action is the same as that necessary for hemolysis. For this purpose the same method as that in the preceeding experiment was employed, and may be outlined as follows:

Leucocytic suspension 0.2 c.c. + heated serum 0.1 c.c. for one hour at 37° C.; centrifugate. Sediment = sensitized leucocytes.

A 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.03 c.c., for one hour at 37° C.; centrifugate.

Supernatant fluid + sensitized leucocytes = leucolysis.

The result might be interpreted as indicating the non-identity of the complement fraction of hemolysin and leucotoxin but it is not final, for even if it were the same, all the complement need not necessarily be bound when the serum is exposed to an erythrocytic suspension. If the result had been no leucolysis, it would have been positive evidence of the identity of the complemental bodies, but the results as found are ambiguous and throw no light upon the question at issue.

Having shown that normal leucotoxins are not the same bodies as the normal hemolysins, the next experiment was to determine whether or not they are specific, or, in other words, is there but one leucotoxin responsible for all the positive results shown in Table 1, or are there a number of different leucotoxins each responsible for leucolysis in one and only one serum-leucocyte combination? The problem can be investigated best by choosing a serum exerting a toxic action on the leucocytes of more than one species, as chicken serum on dog and guinea-pig leucocytes, and determining by means of absorption experiments whether it is the same substance in the serum that causes both leucolyses. If all the dog leucotoxin be removed from a given quantity of chicken serum by saturation with dog white corpuscles and the fluid obtained by centrifugation is still capable of destroying guinea-pig leucocytes, and the reverse likewise obtains, it is evident that the serum contains at least two distinct leucotoxins, one for dog and one for guinea-pig corpuscles, and consequently we would be justified in holding that leucotoxins are truly specific. Such, indeed, is the case, as shown by these experiments.

Dog leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.

Place at 37° C. for 1 hour. Centrifuge.

Supernatant fluid + dog leucocytic suspension 0.2 c.c. = *no leucolysis.*

Dog leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.
Place at 37 C. for one hour. Centrifuge.
Supernatant fluid + guinea-pig leucocytic suspension 0.2 c.c. = *leucolysis.*
Guinea-pig leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.
Place at 37 C. for one hour. Centrifuge.
Supernatant fluid + guinea-pig leucocytic suspension 0.2 c.c. = *no leucolysis.*
Guinea-pig leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.
Place at 37° C. for one hour. Centrifuge.
Supernatant fluid + dog leucocytic suspension 0.2 c.c. = *leucolysis.*

It has been shown that leucotoxin as a whole is bound by leucocytes, but the question was left open as to which of the constituent fractions is bound by the white corpuscles. To settle this point a quantity of normal serum so small that only the complemental fraction was present was treated with leucocytes. After centrifugation the supernatant fluid was added to a quantity of "sensitized" leucocytes (leucocytes + amboceptor [heated serum]) whereupon leucotoxic action took place, showing that the complemental body had not been removed from the normal serum by the leucocytes.

Leucocytic suspension No. 1, 0.2 c.c. + normal serum 0.0015 c.c.
Expose to 37° C. for one hour; centrifugate. and remove fluid.
Leucocytic suspension No. 2, 0.2 c.c. + heated serum 0.1 c.c.
Expose to 37° C. for one hour.
Add centrifugal fluid, and incubate = leucolysis.
Leucocytic suspension No. 3, 0.2 c.c. + normal serum 0.0015 c.c. incubated for one hour = no leucolysis.

Since the thermolabile fraction is not bound by the normal susceptible cells, it must be the thermostabile body that unites with them, and indeed, such can be demonstrated to be the case, for if leucocytes be exposed to heated serum for an hour, removed by centrifugation, and then exposed to a quantity of unheated serum so small that no fixative is present, leucolysis occurs, showing that the amboceptor had been fixed by the leucocytes.

Leucocytic suspension 1 c.c. + heated serum 1 c.c.
Place at 37° C. for one hour; centrifugate.
Sediment + normal serum 0.0015 c.c. = leucolysis.
Leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = no leucolysis.

It is of considerable interest to know if there are bodies in the serum of animals capable of neutralizing or preventing the action of the leucotoxins in foreign sera upon the leucocytes of that animal. To investigate this possibility equal quantities of human and of dog serum (0.2 c.c. each) were added to a suspension of dog leucocytes and incu-

bated at 37° C. for one hour. At the end of that period it was found that leucolysis had occurred. It was realized that this did not afford absolute proof of the non-occurrence of normal antibodies, since it might be that the affinity of the leucotoxin for the cells is stronger than for the antileucotoxin so that when brought in contact with both leucocyte and antibody the poison unites with the former and the presence of the latter is not appreciated. This error was avoided by leaving the human and dog sera in contact with each other for one hour at 37° C. before adding the dog leucocytes to the mixture. At the same time a possible quantitative factor was tested for by employing increasing amounts of dog serum. In no case was leucolysis prevented, showing that normal dog serum, at least, contains no antibody for normal human leucotoxin.

TABLE 4.

ABSENCE OF NORMAL ANTIBODY FOR LEUCOCYTOTOXIN.

Dog Serum	Human Serum	Dog Leucocytic Suspension	Result
....	0.02 c.c.	0.2 c.c.	Leucolysis
0.02	0.02	0.2	"
0.05	0.02	0.2	"
0.1	0.02	0.2	"
0.2	0.02	0.2	"
0.4	0.02	0.2	"

Another important question is how the leucotoxin content of serum is affected by morbid processes in the body, particularly those diseases in which a leucocytosis occurs. For this purpose the sera of a number of patients suffering from such diseases were analyzed quantitatively for leucotoxins by the following method. Dilutions were made and quantities of 0.003, 0.0015, 0.0007, 0.00035 c.c. and in some cases 0.006 c.c. were added to washed dog leucocytes, and the highest dilution showing positive action was taken as the index to the amount of leucotoxin present in the serum. The results are shown in Table 5. While these figures show that the leucotoxic index does not run entirely parallel to the degree of leucocytosis, still it is quite clear that there is some intimate relation between the two, suggesting that leucotoxin either is produced by the hematopoietic tissues or else that it arises from the leucocytes.

It would be interesting to learn whether it is possible to immunize susceptible animals against the leucotoxins of heterologous sera;

further, whether or not normal leucotoxins are the same as the artificial leucotoxins which may be produced by repeated injections of lymphoid tissue.

TABLE 5.

INFLUENCE OF LEUCOCYTOSIS UPON LEUCOCYTOTOXIC INDEX.

Disease	Leucocytosis	Leucotoxic Index
Malaria	None (5,000)	0.003
Pneumonia No. 1	14,000	0.0007
Acute artic. rheumatism	17,000	0.0007
Pneumonia No. 2	21,000	0.0007
Pleurisy No. 1	26,000	0.0007+
Pleurisy with effusion No. 2	26,000	0.00035+
Pneumonia No. 3	28,700	0.0007
Gonorrheal arthritis	28,000	0.00035+
Bronchopneumonia	51,000	0.00035+
Scarlatina No. 1	No count	0.0015
" No. 2	"	0.0015
" No. 3	"	0.00035+
" No. 4	"	0.007
" No. 5	"	0.006
" No. 6	"	0.00035
Hemophilia	"	0.0015
"	"	0.0015
"	"	0.0007
Normal	.....	0.006 to 0.003

The results above obtained may be summarized as follows:

Normal leucotoxins have a fairly wide distribution. They consist of a thermostabile specific body and a thermolabile non-specific substance.

They are distinct from the normal hemolysins, have a different distribution, and are present in considerably smaller amounts of serum.

Their action is essentially upon the nuclei of the leucocytes. They affect all types of leucocytes, but the large mononuclear variety is the most susceptible and the polymorphonuclear form the least susceptible to their action.

Normal sera do not appear to contain antibodies to the leucotoxin of foreign sera.

In diseases associated with a leucocytosis there is an increase in the amount of normal leucotoxin in the serum, this increase corresponding more or less to the extent of the leucocytosis.

In conclusion it is perhaps permissible to point out that, in choosing combinations for experimental opsonic work, proper results can be obtained only by avoiding those in which the serum exerts a poisonous action upon the leucocytes.

I wish to thank Professor Ludvig Hektoen here for his valued advice and suggestions in regard to this investigation.